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14. ABSTRACT

Castration-recurrent prostate cancer (CR-CaP) represents the greatest cause of CaP-associated mortality due to its resistance to standard chemo- and radiotherapies that are efficacious against primary, androgen sensitive (AS)-CaP. CR-CaP progresses to metastatic disease in local lymph nodes and bone in the absence of serum androgen levels. Two recently FDA-approved drugs, abiraterone (ABI) and enzalutamide (ENZ), have shown clinical efficacy against CR-CaP, yet drug resistance readily occurs within months of treatment, severely limiting clinical benefit. This validates these drugs and their targets in CR-CaP, however, knowledge regarding the mechanism underlying ABI- and ENZ-resistance will greatly benefit CR-CaP patients by producing better long-term efficacy of these drugs as well as next generation versions. The current proposal aims at directly dissecting the molecular mechanism underlying ABI and ENZ resistance in CR-CaP. This is based on recent data showing that a shortened variant of the androgen receptor (AR) protein, called AR-V, is directly responsible for malignancy of CR-CaP, and moreover, that ENZ or ABI treatment actually enhances production of AR-V, leading to more drug resistance. We plan to isolate the AR gene sequence responsible for enhancing AR-V production using a novel molecular genetic technique, and then to identify the enhancer-binding proteins that drive enhancer activity, with the long-term aim to prevent ENZ/ABI resistance by interrupting this interaction with small molecule inhibitors. Thus, this project is very timely and novel, and its findings will have a major benefit to CR-CaP patients by identifying pathways to enhance ENZ and ABI targets.

15. SUBJECT TERMS

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Irwin H. Gelman, Ph.D.

INTRODUCTION

Castration-recurrent prostate cancer (CR-CaP) represents the greatest cause of CaP-associated mortality due to its resistance to standard chemo- and radiotherapies that are efficacious against primary, androgen sensitive (AS)-CaP. CR-CaP progresses in the absence of serum androgen levels, yet recent data indicate that it remains driven by full-length (FL) androgen receptor (AR) that responds to low levels of tissue intracrine androgens or splice-variant (V) AR missing at least part of the ligand-binding domain. FDA-approved targeting of intracrine androgen production by the CYP17A1 inhibitor, abiraterone (ABI), or by the AR antagonist, enzalutamide (ENZ), have shown clinical efficacy against CR-CaP. Drug resistance readily occurs, severely limiting clinical benefit: ENZ resistance, typified by the LNCaP tumor cell variant, MR49F, is marked by induction of AR-Vs, whereas ABI resistance is marked by AR-FL or CYP17A1 overexpression. However, little is known about the mechanisms governing drug resistance. This project's hypothesis is that the AR gene locus encodes a cryptic enhancer that drives production of AR-Vs, which, in turn, promote resistance to ABI or ENZ treatment in CR-CaP. Importantly, the characterization of CR-driving enhancer sequences and transcription/splicing factors will help define how CR-CaP becomes resistant to ABI and ENZ through the induction of AR-Vs. Future studies will develop inhibitors of AR-V induction that target the transcription/splicing factors that bind CR-driving enhancers of AR-V production, thereby increasing CR-CaP survival rates by enhancing long-term efficacy of ABI, ENZ, or future AR/CYP17A1 antagonists. Specifically, we propose to identify cis-acting enhancer regions in the AR locus that promote production of AR-V splice variants using a novel NGS-related technique, STARR-seq, and after validating using luciferase reporters, we plan to use DNA/enhancer-pulldown and LC-MS/MS to identify transcription factors that selectively bind these enhancers in order to drive the production of AR-V in CR-CaP cells and especially, after ENZ or ABI treatment. Overexpression or siRNA-mediated knockdown of no more than two of these factors will be tested for the ability to induce or repress AR-V production in either AS- or CR-CaP cells.

KEYWORDS

Prostate cancer, metastasis, androgen receptor, castration recurrence, AR-V7, CYP17A1, enzalutamide, abiraterone, enhancer, STARR-seq, bacterial artificial chromosome, next-gen sequencing, transcription factors

ACCOMPLISHMENTS

The accomplishments are listed below relative to each of the original tasks described in the Statement of Work.

Task 1. Production and validation of enhancer libraries from the AR locus (Months 1-3).
-produce ~600bp sheared DNA fragments from human Y-chromosome BAC clones containing and flanking the AR locus, ligate to Illumina library adapters plus adapters for 5'-AgeI and 3'-SalI sites, splice into STARR-seq Screening Vector. Transform competent *E. coli*, produce plasmid library. Verify fragment coverage and clone frequency by NGS.

Report: We obtained three BAC clones covering ~150Mb of the coding and flanking regions for AR. Two clones were from the RPCI BAC collection ("RP11") and the third (CTD-2563D17) was purchased from BACPAC Resources (Oakland, CA)(see below). The BAC DNAs were fragmented, spliced into Illumina-compatible libraries, and shotgun cloned into the STARR-seq Screening vector. An aliquot of the library was sequenced, as was an aliquot of the total DNA cloned into the STARR-seq vector, indicating that the final STARR-seq clones covered ~93% of the AR locus covered by the BACs. Bacterial libraries were produced after transformation of super-competent *E. coli*. Note that each BAC clone yielded one independent STARR-seq sub-library (i.e.- STARR-seq libraries in total).

AR (androgen receptor)	**rs5919432**	SNP=67,801,708	**Chromosome X:** 67,544,032..67,730,619	RP11-479J1, RP11-963N10, CTD-2563D17

Task 2. Transfect CR-CaP cells with enhancer screening library, select for GFP induction by ENZ or ABI (Months 3-4)

-Transiently transfect CR-CaP cells, split into three aliquots, treat with ABI, ENZ or vehicle overnight. Isolate by FACS ENZ/ABI-enhanced GFP-positive cells. Isolate plasmid DNA, transfect competent *E. coli*, purify plasmids, identify enhancer regions induced by ENZ/ABI in all three CR-CaP lines (C4-2, 22Rv1 and VCaP-CR) by NGS using Illumina primer sets. Map enhancers (NGS read peaks) to AR locus using UCSC Genome Browser.

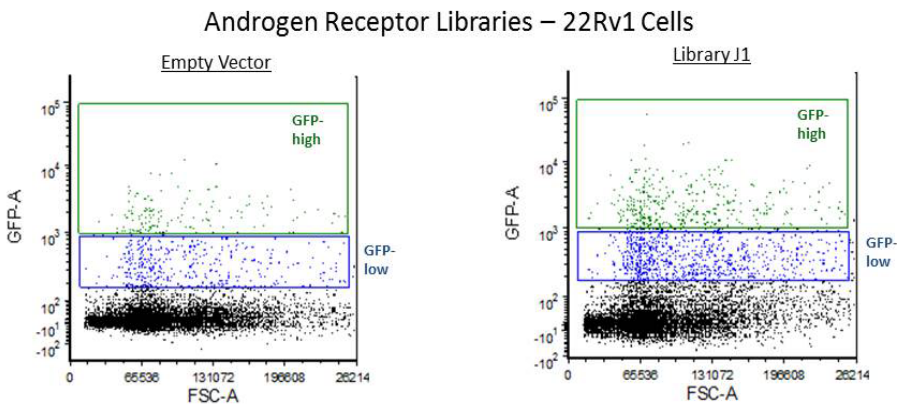

Fig. 1. Cytometric analysis showing increased high and low GFP-expressing cell frequency in 22Rv1 cells transiently transfected with the J1-AR STARR-seq library compared to empty vector alone.

Report: In order to show that the STARR-seq libraries contained potential enhancer regions, 22Rv1 cells (which express full-length AR and AR-V7) were transiently transfected with either empty STARR-seq or the J1 (from BAC clone, RP11-479J1) or N10 (BAC clone RP11-963N10) libraries, and then analyzed for GFP expression after 40 h by either FACS (Fig. 1) or fluorescence microscopy (Fig. 2). Figs. 1 and 2 show that the J1 library produced a significant increase in cells expressing high and low GFP levels compared to empty vector alone. As a control, we showed that 22Rv1 cells have an innately high level of transfectibility in as much as transfection with pEGFP resulted in 20-25% GFP-expressing cells (Fig. 2).

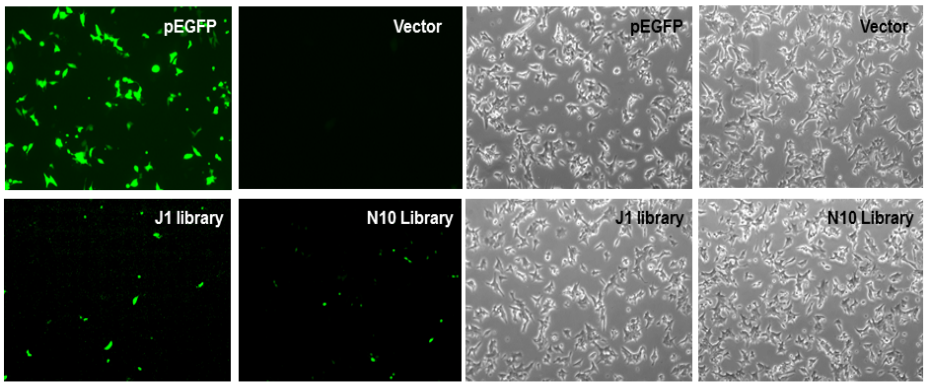

Fig. 2. Fluorescence (left panels) vs. phase-contrast (right panels) microscopic analysis showing increased frequency of GFP-expressing cell 22Rv1 cells after transient transfection with the J1- or N10-AR STARR-seq library compared to empty vector alone. Transfection with pEGFP alone showed that the 22Rv1 cells had a ~20-25% transfection frequency.

Task 3. Validate individual enhancer clones (Months 5-6).

-Transfect individual enhancer clones (in screening vectors) into matched AR- vs. CR-CaP cell lines (e.g.: LNCaP vs. C4-2), or in CR-CaP lines +/- ENZ or ABI treatment, score by cytometry for CR-induced and drug-enhanced GFP expression. Positive enhancer regions will be recombined to a minimal, enhancerless luciferase reporter construct via *att* Gateway recombination sequences that flank the AgeI/SalI enhancer region insert site. After Sanger sequencing to verify the recombinants, the reporters will be transfected as above (into matched AR- vs. CR-CaP cell lines, or in CR-CaP lines +/- ENZ or ABI treatment) to show that the enhancers encode generic transcriptional activity in CR-CaP cells, and enhanced levels in drug-treated CR-CaP cells.

Report: In order to enrich for potential STARR-seq library clones that encoded AR-V7 enhancer activity, we selected for "high" and "low" GFP-expressing 22Rv1 cells transiently transfected with the 3 AR BAC STARR-seq libraries (as shown in Fig. 2). Plasmid DNAs isolated from those sorted cells were purified and used to transform competent *E. coli*. Plasmid DNA was purified from individual bacterial clones (colonies) using a 96-well plates. Individual DNAs from these sub-libraries were transfected into LNCaP vs. C4-2 cells or into 22Rv1 cells, and the 22Rv1 cell pools divided into three sub-pools the next day. These sub-pools were grown in regular media or media supplemented with ENZ or ABI, on the assumption that these drugs would select for the upregulation of AR-V7. We then identified cases in which individual DNAs induced more GFP fluorescence in C4-2 vs. LNCaP cells, or in 22Rv1 cells treated with either ENZ or ABI vs. vehicle, on the assumption that these might encode AR-V&-regulating enhancers. Of the initial 288 clones, 17 induced more GFP in the C4-2/LNCaP comparison, and 22 induced more GFP in 22Rv1 following ENZ or ABI. All these were transferred to the STARR-seq luciferase vector via GATEway recombination.

Task 4. Identify enhancer clones that control AR-V induction (Months 6-7).

-Overexpress by transient transfection the positive enhancer screening clones in LNCaP ($AR\text{-}V^-$), 22Rv1 ($AR\text{-}V^+$) and ENZ-resistant MR49F cells, select by FACS for GFP-positive transfectants, analyze by IB for relative changes in AR-V vs. –FL protein levels.

Report: Plasmid DNAs inducing increased GFP expression in the screens described in Task 3 had their inserts sequenced using Illumina adaptor-specific primers. We identified three potential enhancer sequences, two of which mapped in Intron 6 and one within Intron 3 of the AR gene locus. Although not finished, we are currently scanning these regions for the possible co-mapping of the recently described small eRNA that are transcribed in enhancer areas (Science, 347:1010-1014, 2015).

Task 5. Identify proteins that differentially bind the enhancers (Months 7-10).

-Bind lysates from matched AR- vs. CR-CaP cell lines, or CR-CaP lines +/- ENZ or ABI treatment to AR-V-regulating enhancer regions (biotin-labeled ds biotin-labeled oligonucleotides bound to streptavidin columns), separate bound proteins (after washing) by SDS-PAGE, stain with MS-compatible Deep Purple dye, isolate differentially bound proteins, trypsinize, subject to LC-MS/MS. Validate differential binding by IB analysis of enhancer DNA-pulldown or by ChIP using antibodies specific (and ChIP-validated) for the differentially bound proteins (no more than 2 such proteins will be analyzed).

Report: Starting with a biotin-labeled 5'-primer, we have produced d.s.-DNA fragments (600-800b.p. long) covering all three identified putative AR-V7 enhancers. As a negative control, we produced a similar biotin-labeled fragment from a STARR-seq clone that had no enhancer activity. These DNAs were incubated with cell lysate from C4-2 cells or from 22Rv1 cells treated for 3 days with ENZ. Proteins that bound were sent for LC-MS/MS analysis, with the aim to identify proteins that only bound to the 3 potential AR-V7 enhancers. This Task is not yet fully completed as we are awaiting the MS results.

Task 5. Determine if AR-V-inducing enhancer-binding proteins are sufficient and necessary for AR-V production (Months 10-11).

-If overexpression of enhancer screening clones induced AR-V production in AS-CaP cells (i.e.- removal of a repressor protein) in *Task 4*, then treat AS-CaP with siRNA to the enhancer-binding protein gene. If overexpression of enhancer screening clones reduced AR-V production in CR-CaP cells (i.e.- removal of an inducer protein) in *Task 4*, then overexpress by transient transfection of CR-CaP with an enhancer-binding protein expression vector. Analyze relative AR-V vs. –FL proteins levels by IB as above.

Report: Not yet completed in lieu of the MS results from Task 4.

Task 6. Produce final report (Month 12).

Report: This document constitutes the Final Report.

IMPACT
The current project has identified novel enhancer regions in the AR gene locus that may control the expression of the splice variant, AR-V7, whose expression has been shown to drive resistance to current second-line chemotherapies in CR-CaP, such as ENZ and ABI. We have successfully used the novel STARR-seq cloning system to identify new enhancer regions, mapped these to potential sites of so-called enhancer-RNA (eRNA) expression, and then used these regions to identify possible enhancer binding proteins that might drive the expression of AR-V7. Though the last part of this project awaits full completion and analysis, identification of such enhancer binding proteins should facilitate our ability in future studies to show that expression and/or DNA binding activity of these protein factors is enhanced in CR-CaP, especially after selection of ENZ- or ABI-resistant growth. The long-range goal of this effort would be to identify antagonists of these proteins (their expression and/or activity) so as to prevent the expression of AR-V7, thereby making the clinical effects of ENZ or ABI more durable.

CHANGES/PROBLEMS
Although we were able to perform enhancer oligonucleotide pulldown experiments to identify binding proteins, we had complications on sensitivity at our MS Core lab (SUNY at Buffalo Proteomics Core Lab, Jun Qu, Director). This has delayed our definitive identification of enhancer binding proteins, however, we feel confident that our current analyses will yield usable data.

PRODUCTS
Next-gen sequencing-ready STARR-seq enhancer libraries, potential AR-V7-driving enhancer clones, enhancer binding proteins.

PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS
Roswell Park Cancer Institute: Irwin H. Gelman, Ph.D.- PI
SUNY at Buffalo: Jun Qu, Ph.D., Director, Proteomics Core

SPECIAL REPORTING REQUIREMENTS
None

APPENDICES
None

BIBLIOGRAPHY
None

LIST OF PERSONNEL
Michael Allen: medical fellow (performed the STARR-seq enhancer cloning and validation studies).